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Detection of siRNA-mediated target mRNA cleavage activities in human cells by a novel stem-loop array RT-PCR analysis

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ABSTRACT

The small interfering RNA (siRNA)-mediated target mRNA cleavage activity generates cleaved mRNA fragments with varied termini, which creates major technical challenges for the accurate and efficient detection and verification of cleavage sites on target mRNAs. Here we used a sensitive stem-loop array reverse transcription polymerase chain reaction (SLA-RT-PCR) approach to detect and verify the siRNA-mediated target mRNA cleavage sites by determining precise sequences at the 3′- termini of cleaved mRNA fragments in human cells under physiological conditions. Our results demonstrated the great potential and broad applications of using the SLA-RT-PCR as a sensitive, cost-efficient, and high-throughput tool to systematically detect siRNA-targeted mRNA cleavage sites and fragments in human cells.

1. Introduction

The ability of short-interfering RNA (siRNA) to silence the activity of specific genes has been proved to be very useful in dissecting genetic function [1]. The siRNA also holds considerable promise as a novel therapeutic approach to silence disease-causing genes, particularly those encoding so-called 'non-druggable' targets that are not amenable to conventional therapeutics such as small molecules, proteins, or monoclonal antibodies [2,3]. However, the major obstacles to efficiently knock-down gene expression in vivo and use siRNAs to silence the pathogenic genes in clinical practice by RNAi technologies are the systemic delivery of therapeutic siRNAs [3–5], their off-target effects [2,6], and the lack of accurate and efficient methods and techniques in detection of target mRNA intermediates and end-products resulted from siRNA activities [7,8].

It has been shown that mammalian siRNAs repress gene expression by cleaving target mRNAs and leading to the sequential degradation through RNA-induced silencing complex (RISC) [9,10]. The siRNA cleavage site on the mRNA target is usually located in the middle of the region spanned the siRNA:target duplex [11]. Several reports recently suggested that the efficacy of RNAi observed in vivo could also be resulted from a general transcriptional down-regulation induced by the double stranded structure of siRNA without involving specific target mRNA cleavage [12,13]. Therefore, it is critical to confirm that an observed target gene expression knockdown has occurred through the siRNA-induced mRNA

cleavage. However, the precise cleavage sites on the siRNA-targeted mRNA were not commonly identified and confirmed in current literatures. Such efforts might partly been hampered by technical difficulties in detecting and verifying the cleaved mRNA intermediates and end products. New biological and biochemical methods are much needed to efficiently and accurately identify intermediates and end-products resulted from the siRNA action in multicellular organisms under physiological conditions. In this study, we used a novel stem-loop array RT-PCR (SLA-RT-PCR) assay to accurately identify siRNA-mediated target mRNA cleavage sites by efficiently detecting the cleaved 5′-fragments of target mRNAs under the physiological condition in human cells. We used the synthetic siRNAs with an ectopically expressed mRNA target templates as testing models, and the wild-type (wt) and mutant (mut)-sequence specific siRNAs to endogenous target mRNA to evaluate the sensitivity, specificity, and efficacy of the SLA-RT-PCR assay for detecting siRNA-mediated mRNA cleavage in mammalian cells under physiological conditions. Our results demonstrated the great potential and broad applications of using the SLA-RT-PCR assay to systematically detect intermediates and end products of target mRNA cleavage in multi-cellular organisms under physiological conditions.

2. Materials and methods

2.1. Cell culture and siRNA treatment

The human non-small cell lung cancer cell (NSCLC) lines H1299 (KRAS-wild type) and H358 (KRASG12C (GGT to TGC)-mutant) were obtained from American Type Culture Collection (ATCC).

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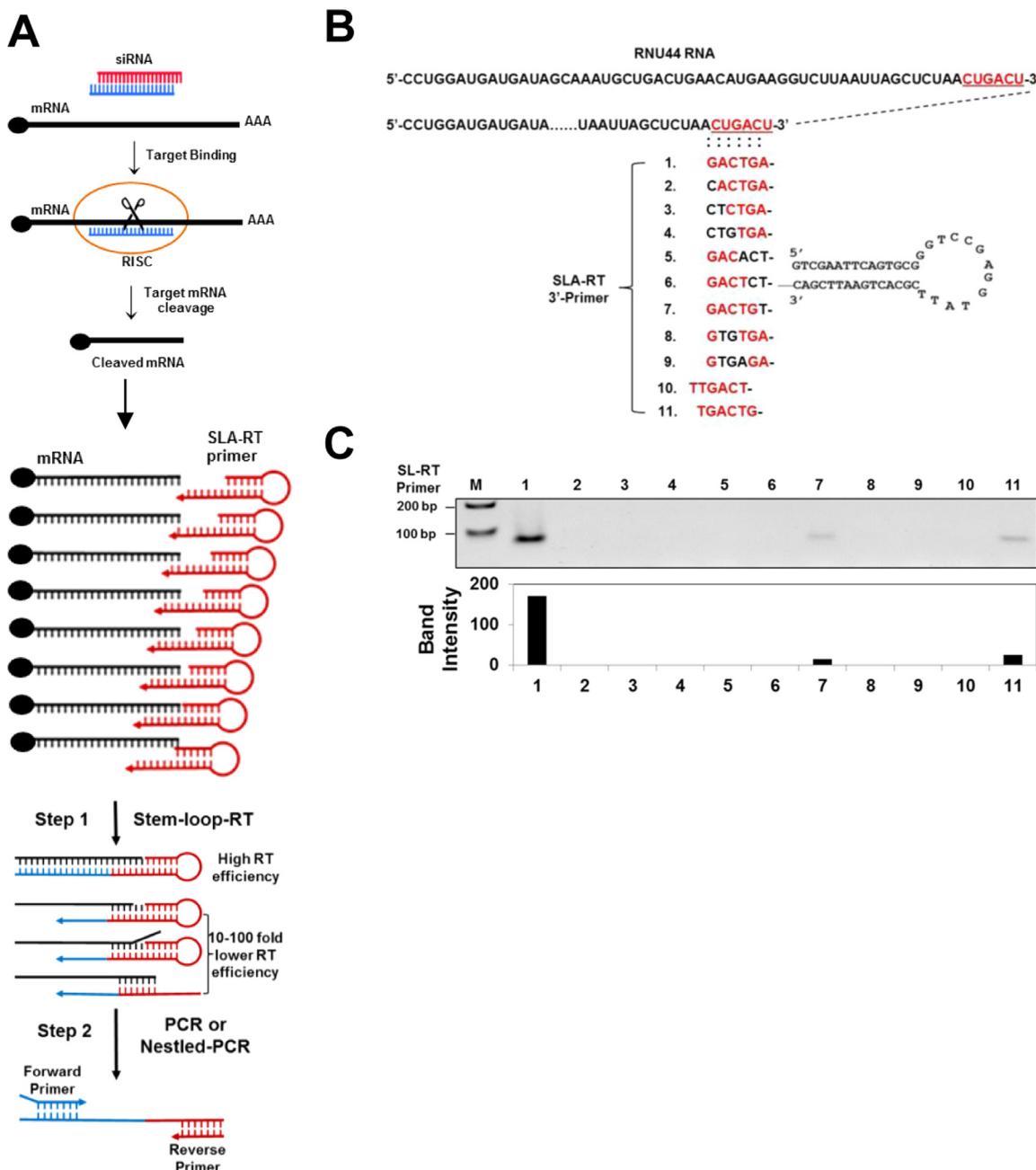
Fig. 1. Schematic representation of detection siRNA-mediated target mRNA cleavage activities by SLA-RT-PCR assay. RNA-induced silencing complex (RISC) cleaves target mRNA into two distinct fragments: a 5′ fragment with a 3′ hydroxyl group, and a 3′ fragment with a 5′ phosphate. (A) SLA-RT-PCR assay includes two steps: SLA-RT and PCR. SLA-RT efficiently detects the cleaved 5′-fragments of target mRNA. PCR is preceded with a pair of forward and reverse primer that contains an antisense sequence complementary to the 5′-end of target mRNA and to the 3′-end of the stem loop of RT-primer. (B) SLA-RT-Primer design and the synthetic RNA44 RNA template sequences for detection and verification of 3′ ends of synthetic small nucleolar RNU44 RNA by SLA-RT-PCR. The predicted full length RNU44 RNA sequences with 3′ end sequences (underlined) targeted by 3′-SLA-RT primers are shown. (C) Detection and verification of 3′ ends of RNU44 RNA by 3′-SLA-RT primers. SLA-RT-PCR products were detected by agarose gel electrophoresis (upper panel) and relative band intensity was shown in lower panel.

H1299 and H358 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum in an atmosphere of humidified air containing 5% CO_2. Cells were grown in a 6-well plate to 80% confluence and then transfected with siRNA. The transfection was performed using the transfection agent DharmaFECT 1 according to the manufacturer's protocols (Dharmacon).

2.2. Plasmid construction

The siRNA-targeted mRNA sequences were directly derived from tumor suppressor gene TUSC2 [14]. The plasmid vector containing the siRNA target sequence was constructed by inserting the siRNA targeting sequence into the reporter GFP Plasmid (Fig. 3A). All sequences in plasmid constructs were confirmed by automated DNA sequencing.

2.3. Synthesis of materials

The small RUN44 RNAs with a predicted sequence of sixty-six nucleotides (Fig. 2A) were synthesized using commercial source (Sigma) and purified by polyacrylamide gel electrophoresis (PAGE). The purified RUN44 RNA was used as a RNA template to evaluate SLA-RT-PCR for detection and verification of the 3′ end of RUN44 RNA. All SLA-RT primers and PCR primers and siRNAs were

synthesized and purchased from Sigma and their corresponding sequences were described and illustrated in individual figures.

2.4. RNA isolation

Cells were grown on 6-well plates and approximately 2×10^6 cells were used to isolate the total RNA using the TRIzol reagent (Life Technologies) and the additional phenol/chloroform extraction was performed before ethanol precipitation according to the manufacturer's protocol. The isolated total RNA was stored in 70% ethanol at -80 °C. The purified total RNA was dissolved in RNase-free water for SLA-RT-PCR reactions.

2.5. Stem-loop array reverse transcriptase reaction (SLA-RT)

RNA samples were briefly treated with 0.04U/µl RNase-free DNase I. RNA was reversed transcribed using the High Capacity Reverse Transcription kit (Life Technologies) in combination with an array of stem-loop RT primers. The 20 µl of RT reaction contained 50 ng of total RNA, 5×10^{-12} mol of SLA-RT primer, 2 µl of

$10 \times$ RT buffer, 1 µl of Multiscribe Reverse Transcriptase, and 0.8 µl of 100 mM dNTPs. To increase reverse transcription efficiency, a pulsed RT reaction was performed on a DNAEngine Peltier Thermal Cycler (Bio-Rad) with 60 cycles at 20 °C for 1 min and 37 °C for 1 s, followed by 60 cycles at 18 °C for 1 min and 37 °C for 1 s, and a final cycle at 37 °C for 30 min, 42 °C for 20 min, and extension at 85 °C for 10 min, and then held at 4 °C.

2.6. 5′RACE

5′-RACE was performed using the 5′ RACE System for Rapid Amplification of cDNA Ends, version 2.0 kit (Invitrogen, Life Technology) with the manufacturer's instructions. Briefly, first-strand cDNA was synthesized using a kras -specific primer (5′-TCA-CATTTATTTCCTACTAGGACCATA-3′). The original mRNA template was removed by treatment with RNase Mix. Unincorporated dNTPs, GSP1, and proteins were separated from cDNA using a S.N.A.P. Column. A homopolymeric tail was then added to the 3′-end of the cDNA using TdT and dCTP. PCR was amplified by GSP2 primer (5′-GTACATCTTCAGAGTCCTTAACTCTT-3′) and deoxyinosine-
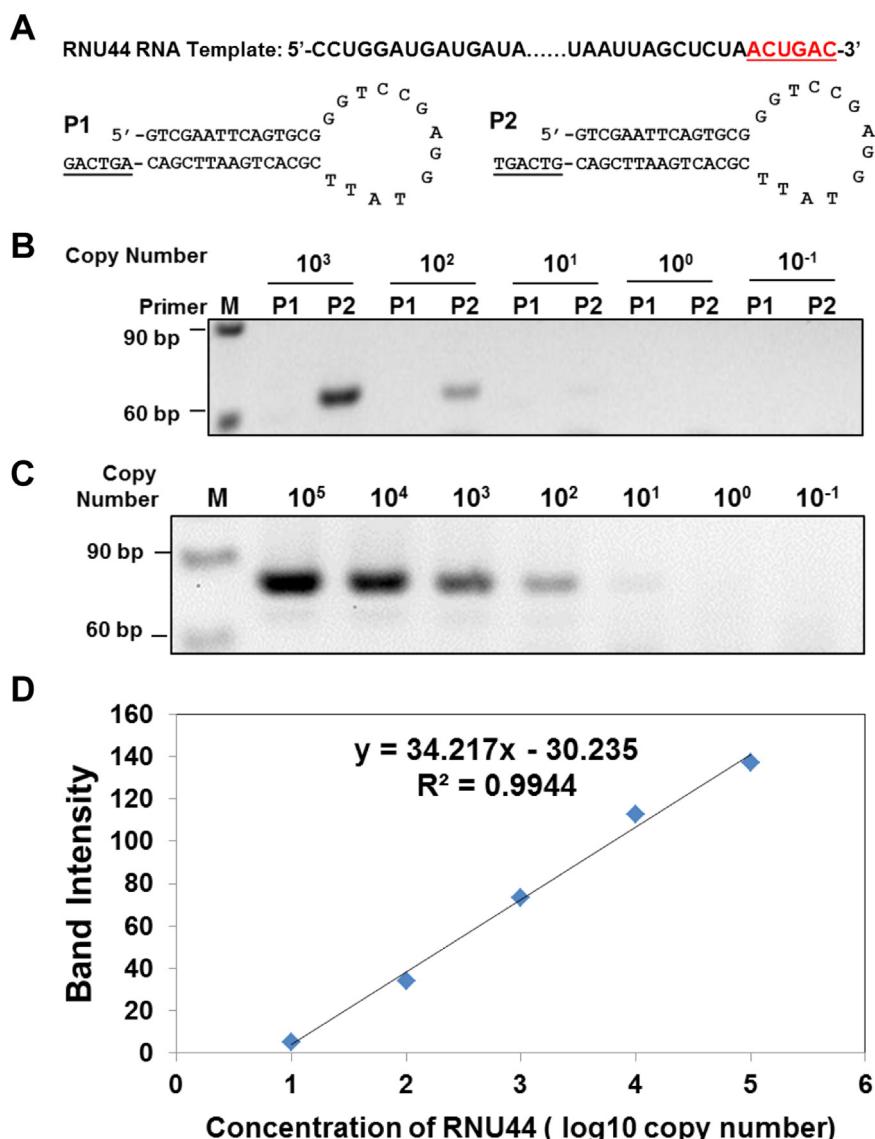

Fig. 2. Sensitivity of the SL-RT-PCR assay. The SL-RT-PCR reactions were performed using two SL-RT primers (P1 and P2) (A). Known concentrations of input synthetic RNU44 RNA templates over five orders of magnitude from 10^{-1} to 10^3 molecules and RT-PCR products were analyzed by agarose gel electrophoresis (B). (C) Dynamic range of the SL-RT-PCR assay. From 10^{-1} to 10^5 molecules of synthetic RNA templates were detected by using SLA-RT-PCR assay and SL-RT-PCR products were analyzed by agarose gel electrophoresis (C) and corresponding liner regression plots of the RNA template input vise the relative output of SL-RT-PCR products are presented (D).
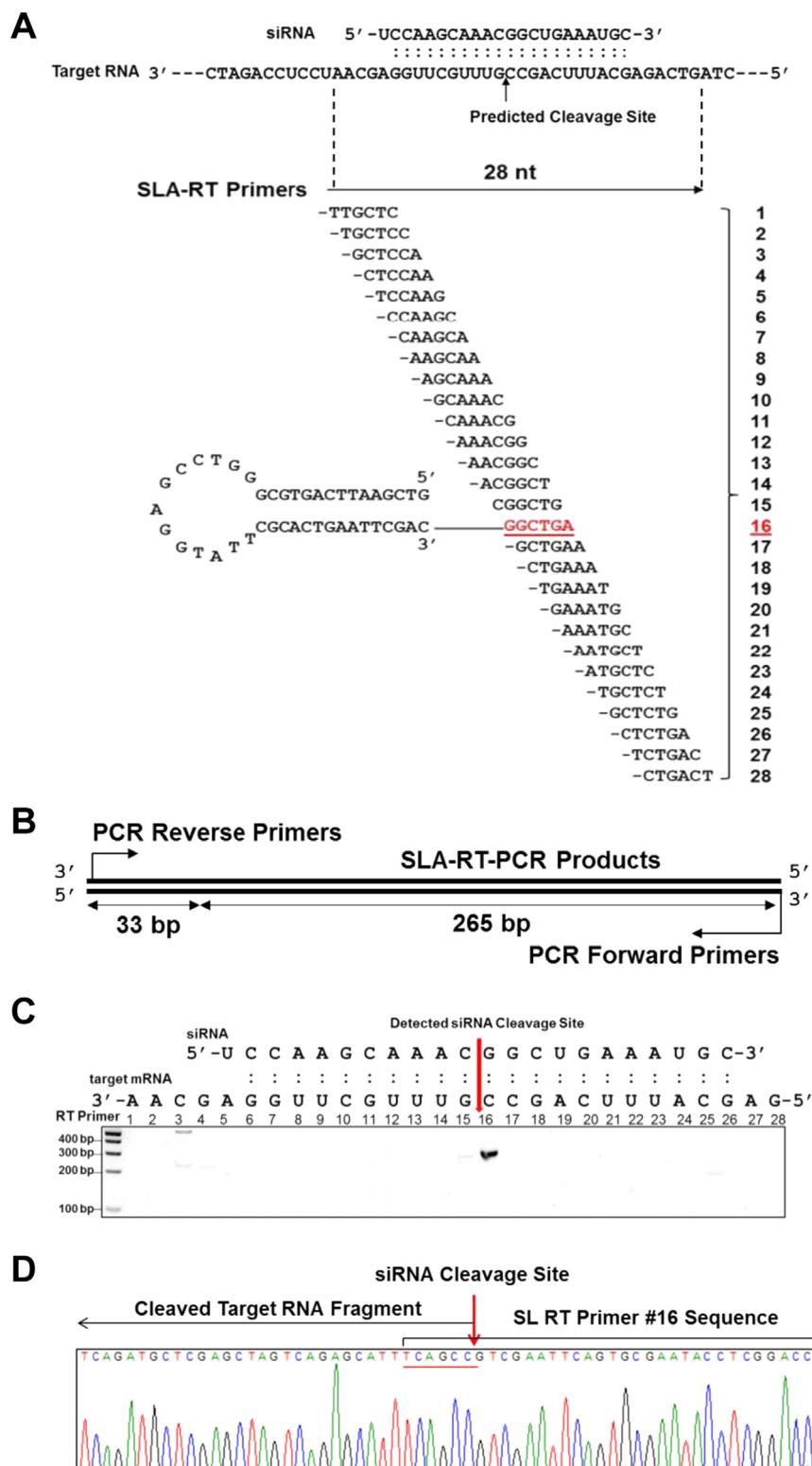
Fig. 3. Detection of siRNA cleavage sites by SLA-RT-PCR assay. (A) Sequences of siRNA and its target mRNA and SLA-RT primers. The sequences of the SLA-RT primer #16 anticipated for most efficiently detecting the predicted siRNA cleavage site on target RNA template is underlined. (B) The predicted size of SLA-RT-PCR fragment amplified by SLA-RT primer #16. (C) Detection of siRNA cleavage site in H1299 cells co-transfected with siRNA- and target RNA expressing plasmids by SLA-RT-PCR. (D) DNA sequencing confirmation of the siRNA cleavage site and the cleaved RNA fragment detected by SAL-RT-PCR.

containing anchor primer with cycling as described in the kit manual: 1 cycle of 94 °C for 4 min, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s 72 °C for 1 min, and final extension of 72 °C for 10 min. From this reaction, 10 μl was analyzed on a 1.5% agarose gel.

2.7. Polymerase chain reaction (PCR)

Each cDNA sample generated from the SLA-RT reaction was diluted 500-fold. Twenty μl **of** PCR mix contain 1 μl of diluted RT products, 2 μl of 10 × standard PCR buffer, 0.8 μl of 10 mM dNTPs,

0.5 μl of 100 mM MgSO₄, 0.2 μl of Taq polymerase, 1 μl of 10 μM of forward primer and 1 μl of 10 μM of reverse primer. PCR reactions were conducted at 95 °C for 5 min, followed by 35 cycles at

95 °C for 15 s, 61 °C for 30 s, and 37 °C for 30 s, and a final extension at 72 °C for 10 min, on DNAEngine Peltier Thermal Cycler.
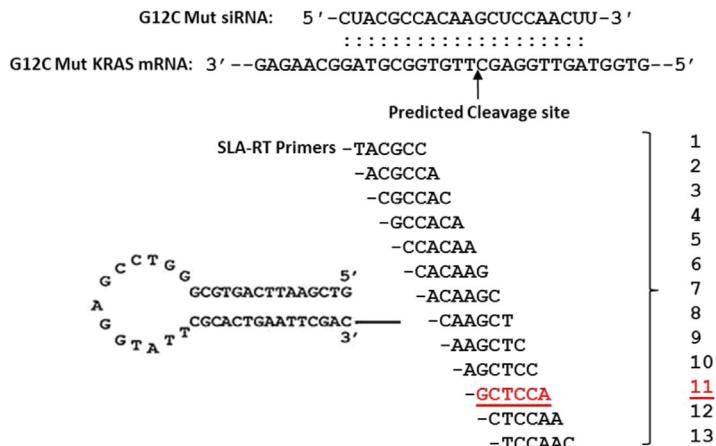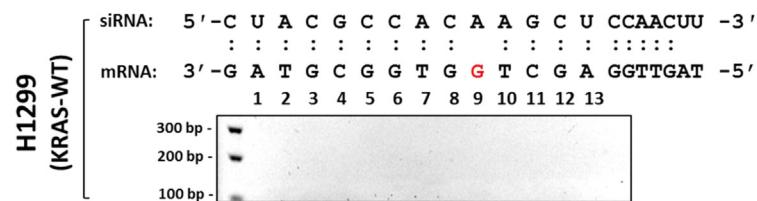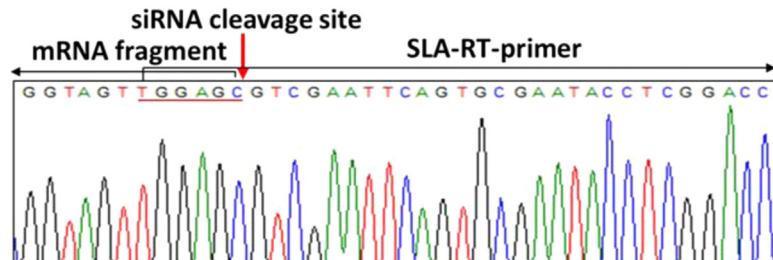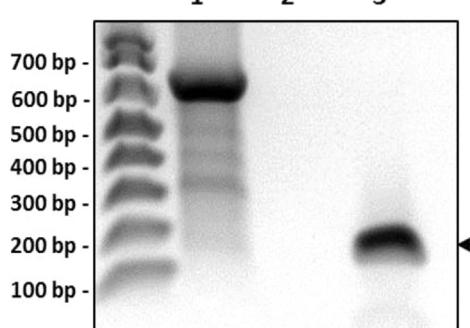
The nucleotide sequences of the PCR primers are as followings:

U6: sense primer, 5′-CCTGGATGATGATAAGCAAATGC-3′, antisense primer, 5′-GTGCGGGTCCGAGGTATTC-3′.

TUSC2: sense primer, 5′-CGGCATGGACGAGCTGTACAAGTA-3′, antisense primer, 5′-GTGCGGGTCCGAGGTATTC-3′.

KRAS: sense primer, 5′-GAAGGTGGCGGCGGCTCG-3′, antisense primer, 5′-GTGCGGGTCCGAGGTATTC-3′.

PCR products sequence analysis was performed on an ABI 3730 DNA sequencer by DNA Analysis Core Facility at MD Anderson Cancer Center.

2.8. Agarose gel electrophoresis

SLA-RT-PCR products were analyzed by 1.5% agarose gel electrophoresis in $1 \times$ Tris-Borate-EDTA (TBE) buffer containing 89 mM of Tris Base and 89 mM of Boric Acid. Electrophoresis was performed at 100 V for 60 min. Gel was stained in ethidium bromide bath for 10 min prior to visualization using a UV transilluminator.

3. Results

3.1. Detection and verification of 3′ ends of synthetic RNU44 RNA by SLA-RT-PCR

We adopted a novel SLA-RT-PCR technique, originally developed by Chen et al. [15], to systematically verify and detect intermediate mRNA fragments cleaved by siRNAs. This approach accurately identified unique nucleotide ends generated by the siRNA-mediated target cleavage activities in human cells. The principle of works, the experimental design, and assay procedures are illustrated in Fig. 1A. The SLA-RT-PCR assay includes two steps: SLA-RT and PCR. SLA-RT primers comprised two unique sequence components: a short stretch of 6-base single-stranded nucleotides at the 3′ end of the primer sequences that are complementary to the 5′-terminal sequences of the target mRNA, and a double stranded stem at the 5′ end that forms a stem-loop to function as a forceps to stabilize the secondary structure of the primer. The terminal sequences of the siRNA-cleaved target mRNA fragments are specifically recognized by a six-nucleotide complementary extension at the 3′ends of the RT-primer array, which could efficiently prime the reverse transcription of the cleaved mRNA fragments into cDNAs. The uses of an array of stem-loop RT primers allow accurate recognition and verification of unique ends of the mRNA fragments processed by endonucleases. If the SLA-RT primers have no perfect match, on both the length and the base, to the sequence of the 3′ or 5′ end of the mRNA fragment the efficiency of RT reaction would have been dramatically reduced. The subsequent PCR is preceded with a pair of forward and reverse primer that contains an antisense sequence complementary to the 5′-end of mRNA and to the 3′-end of the stem loop of RT-primer, respectively. The abundance of the transcript-specific SLA-RT-PCR amplicons represents the distribution of cleaved RNA fragments and is determined by agarose gel electrophoresis. The success of SLA-RT-PCR relies deeply on the differential priming efficiency between matched and mismatched SLA primers with the cleaved mRNA fragments at 3′ termini. Thus, the priming efficiency by SLA-RT-primers is determined by the perfect match, mismatch, overhang, and gap presented in SLA-RT-primer: mRNA target hybrids.

We first used the synthetic small RUN44 RNA as a model system to evaluate SLA-RT-PCR for detection and verification of the 3′ end of RUN44 RNA fragments (Fig. 1B). The RNU44 is a member of abundant intronic small-nucleolar RNAs that are frequently used as reference genes for measuring endogenous miRNA expression. We designed eleven SLA-RT-primers (Fig. 1B) and performed SLA-RT-PCR reactions using the PAGE-purified synthetic RNU44 RNA as a template to characterize effects of mismatch, overhang and gap in primer/target hybrids on SLA-RT priming fidelity and efficiency. Eleven SLA-RT-primers that differed by at least one nucleotide at 5′ or 3′ end of probe were used to reverse transcript the same RUN44 RNA template. The resultant SLA-RT-PCR amplicons were analyzed by agarose gel electrophoresis (Fig. 1C). As expected, the SLA-RT primer (Primer 1) with the perfect match to the 3′ end of RNU44 template successfully initiated reverse transcription of template RNA with the highest efficiency (Fig. 1C, Lane 1). However, those probes with 2 or more nucleotide mismatches failed to initiate cDNA synthesis even with up to 500 copies of templates incubated. The RT-priming efficiency for probes with single nucleotide mismatch varied with the location of mismatches. The priming efficiency of SLA-RT primers was markedly reduced by the mismatch at the 3′-ends of probes but less sensitive to the mismatch at 5′-sites of probes (Fig. 1C, Lane 7 compared to Lane 1). A similar trend was also observed with a single nucleotide hangover at 5′ end of the probe as demonstrated in Fig. 1C, Lane 11. The priming fidelity of the SL-RT primer was fully restored with a two-nucleotide overhang, as shown in Fig. 1C, Lane 10. These results indicated the high specificity of the designed SLA-RT primers to the 3′ end sequence of RNA fragments.

3.2. The dynamic range and sensitivity of SLA-RT-PCR

To evaluate the dynamic range and sensitivity of the SLA-RT-PCR assay, we compared the SLA-RT-priming efficiency between a gap-generating SLA-RT-primer (P1) and a matched SLA-RT-primer (P2) on the synthetic RNU44 RNA template (Fig. 2A). The gap generated between the RNA template and the primer P1 effectively prevented the reverse transcription of the mismatched template, whereas the RNA template-matched probe P2 could efficiently initiate RT and detect RNA templates at concentrations as low as 10 copies RNA (Fig. 2B). Furthermore, we determined the dynamic range and sensitivity of SLA-RT-PCR assay using SLA-RT primer P2 with the PAGE-purified synthetic RNU44 template in a series of dilution over a range of 7 orders of magnitude from 10^{-1} to 10^5 copies (Fig. 2C). The SLA-RT-PCR assay showed an excellent linear output of PCR products corresponding to the RNA template copies over a dynamic detection range at lease of five orders of magnitudes (from 10 to 10^5 copies) and demonstrated an extremely high sensitivity that is capable of detecting as few as 10 template molecules in SLA-RT-PCR reactions (Fig. 2D). Together, these results clearly demonstrated the specificity and high sensitivity of the SLA-RT-PCR assay.

Fig. 4. Detection of the cleavage site on endogenous mutant KRAS$^{G12C\ (GGT\ to\ TGT)}$ mRNA mediated by the mutant-specific siRNA with SLA-RT-PCR in NSCLC cells. (A) The design of SLA-RT primers for detecting the cleavage site on the mutant KRAS$^{G12C\ (GGT\ to\ TGT)}$ mRNA targeted by mutant-specific siRNA. The SLA-RT primer #11 (underlined) was anticipated to most efficiently detect the predicted siRNA cleavage site on mutant KRAS mRNA template. Detection of the siRNA-targeted cleavage on the endogenous KRAS mRNA in the wt-KRAS-containing H1299 cells (B) and the mutant KRAS$^{G12C\ (GGT\ to\ TGT)}$-containing H358 cells (C) transfected with KRAS$^{G12C\ (GGT\ to\ TGT)}$-specific siRNA by lipofectamine, respectively. After 48 h transfection, total RNAs were isolated and SLA-RT-PCR was performed. The predicted mRNA cleavage fragments (202 bp) should be detected by agarose gel electrophoresis in H358 (KRAS-mut) cells but not in H1299 (KRAS-wt) cells. (D) The KRAS$^{G12C\ (GGT\ to\ TGT)}$-siRNA cleaved KRAS mRNA fragments and cleavage site in H358 cells were confirmed by automated DNA sequencing. The SLA-RT primer probe #11 was marked by red underline. (E) Comparison of SLA-RT-PCR and 5′ RACE assays in detection of KRAS$^{G12C\ (GGT\ to\ TGT)}$-siRNA cleaved KRAS mRNA fragments and cleavage site in H358 cells. The same amount of total RNAs isolated from H358 cells as described in (C) was used as the RNA template for RT reactions initiated by both the 5′RACE and the SLA-RT-PCR assays. The PCR products were analyzed by agarose gel electrophoresis and shown in **Lane 1**, the positive control for 5′RACE method, **Lane 2**, the mut-KRAS-mRNA-specific 5′RACE, and **Lane 3**, the mut-KRAS-specific SLA-RT-PCR assay with SLA-RT primer #11 as described in (A). The KRAS$^{G12C\ (GGT\ to\ TGT)}$-siRNA-cleaved mRNA fragments (202 bp) detected by SLA_RT-PCR is indicated by the arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Detection and verification of siRNA-mediated mRNA cleavage sites by SLA-RT-PCR

In order to evaluate the potential of SLA-RT-PCR assay in identifying siRNA targets and determining its mechanism of action, we used a synthetic siRNA and ectopically expressed artificial mRNA template as a model system to precisely detect and verify the intermediates and end products of the siRNA activity in mammalian cells under physiological conditions. It has been shown that mammalian siRNAs silence gene expression by cleaving their target mRNAs for sequential degradation [10]. The siRNA cleavage site on the mRNA target is usually located in the middle of the region spanned by the siRNA:mRNA duplex, following a canonical pattern of 10 bp from the 5′-end of siRNA in the RISC [11]. The RISC-associated argonaute 2 (AGO 2) cleaves target mRNA into two distinct fragments: a 5′ fragment with a 3′ hydroxyl group, and a 3′ fragment with a 5′ phosphate [16–18]. The siRNA and the siRNA-targeted mRNA template expression plasmid were co-transfected into human non-small cell lung cancer (NSCLC) H1299 cells (Fig. 3). The siRNA and its target mRNA sequences, the predicted siRNA cleavage site, and the designed set of twenty-eight SLA-RT primers for detection of the potential siRNA-directed target RNA cleavage sites were illustrated in Fig. 3A and B. The siRNA-mediated cleavage site on the mRNA target was precisely detected by the SLA-RT-PCR in the middle of siRNA target region at the predicted position "G", which is 10 bp from the 5′-end of siRNA duplex, as shown in Fig. 3C and indicated by arrow. The identity of the SLA-RT-PCR products was further verified by DNA sequencing (Fig. 3D). These results clearly demonstrated the potential applications of SLA-RT-PCR in identifying authentic siRNA targets by precisely detecting and verifying the cleavage sites on target mRNAs mediated by siRNAs in mammalian cells.

3.4. Detection of siRNA-mediated cleavage activities on endogenous mRNA targets

To further demonstrate the utility and specificity of SLA-RT-PCR in detection the siRNA-mediated cleavage sites on endogenous mRNA targets in human cells under physiological conditions, we determined cleavage activities mediated by synthetic siRNAs that specifically target the wild-type and the mutant sites, respectively, on the endogenous KRAS mRNA sequences in human NSCLC H1299 (KRAS-wt) and H358 (KRAS$^{G12C\ (GGT\ to\ TGT)}$-mut) cells (Fig. 4). The mut-KRAS-specific siRNAs (Fig. 4A) were transfected into H1299 and H358 cells by DharmaFect1 transfection reagent and total RNAs were isolated 48 h after transfection. A set of SLA-RT primers covering entire siRNA complementary and the immediately adjacent 5′- and 3′ sequences (Fig. 4A) on wt-KRAS (Fig. 4B) or mut-KRAS (Fig. 4C) mRNA sequences were used to detect and verify the predicted cleavage site as indicated by arrow (Fig. 4A). The siRNA-cleaved KRAS mRNA fragments with a predicted size of 202 bp could be detected by agarose gel electrophoresis only in mut-KRAS$^{G12C\ (GGT\ to\ TGT)}$-containing H358 cells (Fig. 4C) but not in wt-KRAS-expressing H1299 cells (Fig. 4B) transfected by the mut-KRAS-siRNA. The KRAS$^{G12C\ (GGT\ to\ TGT)}$-siRNA cleaved mut-KRAS mRNA fragments and cleavage site in H358 cells were confirmed by automated DNA sequencing (Fig. 4D).

To further validate the utility, specificity, and sensitivity of SLA-RT-PCR assay in detection of siRNA-cleavage site and cleaved 5′-mRNA fragments, we performed a parallel comparison between the SLA-RT-PCR and the 5′RACE (Invitrogen, Life Technologies) methods (Fig. 4E), using the same total RNAs isolated in H358 cells transfected with KRAS$^{G12C\ (GGT\ to\ TGT)}$-siRNA as described in Fig. 4C as the template for RT reactions respectively initiated by the SLA-RT and 5′RACE-RT primers specific to endogenous KRAS mRNA

transcripts. The predicted 5′RACE product was clearly detected by the positive control provided in 5′RACE kit (Fig. 4E, Lane 1) but no 5′RACE products were detected using KRAS-specific RT primer (Fig. 4E, Lane 2). The predicted KRAS$^{G12C\ (GGT\ to\ TGT)}$-siRNA-cleaved mRNA fragments (202 bp) was clearly detected by SLA_RT-PCR using the mutant KRAS-specific SLA-RT primer #11 (A), as similarly shown in (C) and indicated by the arrow. These results demonstrated the sensitivity and advantages of SLA-RT-PCR assay in detection of specific sites and 5′-fragments cleaved by siRNA activities under physiological conditions over the conventional methods such as the 5′RACE.

4. Discussion

siRNA has been shown to be a powerful tool for analyzing gene function and developing novel therapeutic drugs targeting specific disease-causing or cancer-driving genes by silencing expression of a specific gene and encoded protein product [4]. However, for the successful application of siRNA as a therapeutic for human disease, one of most critical questions need to be answered in the clinical practice is to detect and confirm whether the designed siRNA therapeutic hits its predicted gene target. This question can be effectively addressed by accurately detecting the siRNA cleavage sites and cleaved products of the target mRNA. However, such efforts might partly be hindered by technic difficulties in detecting and verifying specific cleavage mRNA sites and the cleaved mRNA intermediates and end products.

Traditional RT-PCR cannot detect mRNA transcript termini and intermediates generated by various cellular processes and biological activities, which are important for understanding the biological significance of mRNA polymorphisms and mRNA functionalities. These mRNA variants might reflect the difference of abundances between full length transcripts and shortened transcripts, produced by partial degradation, modification or cleavages of transcripts through various RNA editing mechanisms, including siRNA targeted mRNA cleavages. Currently, 5′-RACE (rapid amplification of cDNA ends), 3′-RACE, cRT-PCR (circulative RT-PCR) and Next Generation of Sequencing (NGS) are a few technologies to fulfill these needs for mRNA cleavage activities through RNA self-circulation or adapter addition by RNA ligase [19,20]. However, those technologies have their corresponding limits in sensitivity, accuracy, and cost- and time-efficiency. Moreover, the inevitable use of RNA ligase in some of those methods result in enzymatically biased interpretation in the distribution of RNA terminus and significantly reduced their detection sensitivity and accuracy, as we demonstrated in parallel comparison of the SLA-RT-PCR and 5′ RACE methods.

In this study, we have evaluated the sensitivity and specificity of SLA-RT-PCR method for identifying siRNA-mediated target mRNA cleavage sites by accurately detecting the termini of siRNA-cleaved target mRNA fragments on both the ectopically expressed in human cells under physiological conditions. Our results demonstrated a great differential RT-priming efficiency between matched and mismatched SLA-RT-primers to the 3′-termini of siRNA-cleaved target mRNA fragments. This method is extremely sensitive, allowing detection of RNA fragments as few as 10 template copies with a linear correlation up to 5 orders of magnitudes with primers tested. Although the liner correlation could be altered by the different compositions among SLA-RT-primers, which could potentially alter the Tm (melting temperature) of SAL-RT primers, the intensity of the individual SLA-RT-PCR amplicon was principally determined by the RNA fragment abundance and the base-stacking enhancement of perfectly matches between RNA terminal and SLA-RT-primer sequences. The high GC- or AT- rich sequence contents at the 3′-termini of the cleaved target miRNA

fragments may affect the priming efficiency of SLA-RT, however, our testing results with varied base compositions at 3′-termini of synthetic mRNA templates and corresponding matched or unmatched SLA-RT primers demonstrated that the priming efficiency was more depended on the degree of base matches than the content of bases. The SLA-RT-PCR could potentially be used to more quantitatively determine the cleaved mRNA products by real-time PCR with probes specific to the predicted PCR amplicons. This method could also be modified to detect the cleaved mRNA fragments with unknown ends such as 3′-urydylation, tailing, and trimming by modifying the SLA-RT primers with "U-tract" and randomizing 6-nt short stretch at the 3′-end of SLA-RT primers. Due to the high sensitivity and specificity of SLA-RT-PCR as demonstrated, this method is principally sufficient and capable to detect the precise siRNA cleavage site on the targeted mRNA by taking advantages of the high fidelity of SLA-RT primers in recognizing specific ends of RNA fragments generated in RISC and efficiently capturing an immediate and unmodified cleavage fragment from a population of specific-siRNA-cleaved mRNA intermediates and end products at any given time under a physiological condition. By incorporating randomized stem-loop-array RT primer sequences and in combination with targeted deep sequencing, the modified SLA-RT-PCR method can be used as a "high-throughput" assay to capture all potential mRNA cleavage sites and cleaved fragments by "on-target" and "off-target" activities of a specific siRNA.

Our results demonstrated the great potential and broad applications of using the SLA-RT-PCR as a sensitive, specific, and cost-efficient tool to identify authentic siRNA-cleavage sites in multicellular organisms under physiological conditions. SLA-RT-PCR can efficiently detect and confirm whether the designed siRNA therapeutic hits its predicted gene target and could be a useful tool for facilitating the application of siRNA-based therapeutics in human diseases.

Conflict of interests

The authors declare that they have no conflict of interest.

Acknowledgements

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.02.012.

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